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Microbial Production of L-Glutaminase and L-Asparginase from *Aspergillus cervinus* by Submerged Fermentation-*"Potential Anticancer Agents"*

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Abstract—*Cancer cells, especially Acute Lymphoblastic Leukemia (ALL) cells cannot synthesize L-glutamine and hence demand for large amount of L-glutamine for its growth. The same mechanism is followed in L-asparagine synthesis. The use of amidases deprives the tumor cells from L-glutamine/L-asparagine and causes selective death of L-glutamine/L-asparagine dependent tumor cells. Aspergillus cervinus was selected for production of these two enzymes L-asparaginase and L-glutaminase. Submerged fermentation was carried out with two different substrates, Glutamine and Asparagine. Both the enzymes, L-Glutaminase and L-Asparaginase, were assayed in each flask. With Asparagine as substrate, activity of L-asparaginase was 5.08U/ml and L-glutaminase activity was 8.733U/ml. With Glutamine as substrate L-asparaginase activity was 16.65 U/ml and L-glutaminase activity was 47.38U/ml. The study was concluded with result that A.cervinus can be used for large production of these anti cancerous enzymes.*

Keywords*: L-Glutaminase, L-Asparginase, Aspergillus cervinus, submerged fermentation.*

1. INTRODUCTION

Ever since the discovery of cancer, there has been tremendous and repetitive attempts to find out different and safe alternatives for treatment of cancer [3,5,7,9,10,11]. L-Glutaminase can bring about degradation of L-glutamine and thus can act as possible candidate for enzyme therapy. Same in case of L-Asparaginase. L-asparaginase and L-glutaminase have been two effective and efficient enzymes in the treatment of cancer specially in ALL [11,13]. Hence they have been produced by a large number of microorganisms like *Aspergillus flavus* [4], *Streptomyces avermitilis* [6], *Erwinia carotovora* [7], *Pseudomonas aeruginosa* 50071 [8], *Pseudomonas aeruginosa* BGNAS-5 [9], *Penicillium species* [10], Actinomycetes [12], *Aspergillus oryzae* NRRL 32657 [14] , *Aspergillus terreus* [15], *Proteus vulgaris* [16], *Bacillus subtilis* strain hswx88 [17], *Pectobacterium carotovorum*[18], *Aspergillus cervinus* [11] etc. These enzymes exploit the inability of the cancerous cells of synthesize asparagine or glutamine as they are non essential amino acids [1,3,5,11]. L-asparaginase and L-glutaminase deprives the cancerous cells of these amino acids by degrading them and hence causing the death of cancerous cells[5,13]. Recent research efforts have focused on process development (optimization) and scale up of microbial enzyme production. The present study was carried out to produce L-glutaminase and L-Asparginase enzymes from fungal strains

2. MATERIALS AND METHODS

2.1. Materials

L-asparagine and L-glutamine (Kemphasol) was used. A*spergillus cervinus* was procured from NCIM, Pune.

2.2. Plate screening

Primary screening was carried out as reported by Gulati et al[19]. The standard culture was pour plated using Czapek Dox Agar incorporated with phenol red (0.009%), L-asparagine (0.5%). Upon incubation for 2-3 days, the fungal colonies which has shown pink zone were selected as Asparaginase producers. The strain was plated on Czapek Dox agar incorporated with phenol red as indicator, to reconfirm their ability to produce L-asparaginase.

Modified Czapek Dox's (mCD) medium pH 6.2, used for fungi contained 0.2%

(w/v) glucose, 1% (w/v) L-asparagine, 0.152% (w/v) K2PO4, 0.052% (w/v) KCl, 0.052% (w/v) MgSO4.7H2O, 0.003% (w/v) CuNO3. 3H2O, 0.005% (w/v) ZnSO4.7H2O, 0.003%

(w/v) FeSO4.7H2O, 1.8% (w/v) agar, initial pH 6.2 was supplemented with 0.009% (v/v) phenol red as indicator. Control plates were mCD medium containing NaNO3 as nitrogen source instead of asparagine/glutamine. The plates were incubated at 30°C for 48 h. The isolates that showed pink zone around the colonies indicated L-asparaginase production and were selected for determination of enzyme activity.

A 2.5% stock of the phenol red indicator was prepared in 70% ethanol and the pH was adjusted to 7.00 using 0.1 mol/L NaOH [19]. The stock solution of dye was added to 100 ml of modified Czapek Dox's agar medium to get final dye concentrations of 0.001 - 0.009 mg/L [19]

2.3. Production in Submerged fermentation

Submerged fermentation was carried out with *Aspergillus cervinus* with asparagine. Production of L-asparaginase was carried out in Erlenmeyer flask containing Czapek Dox broth as production medium for 7 days at 150 rpm and 37ºC. A loop full of culture was used. Enzyme activity was determined every 24 h. by withdrawing 5 mL of sample. Protein content was also determined by lowry's method. Asparagine was replaced by glutamine to produce L-glutaminase.

2.4. Assay of L-asparaginase and L-glutaminase:

L-Asparaginase was assayed as reported by Imada et al [2]. A reaction mixture containing 0.5 ml of 0.04M L-asparagine, 0.5 ml of 0.5M buffer, 0.5 ml of an enzyme preparation, and distilled water to a total volume of 2.0 ml was incubated at 37 ^{0}C for 30 min. The reaction was stopped by adding 0.5 ml of 1.5 M-trichloroacetic acid. Blank tubes were run by adding the enzyme preparation after the addition of trichloroacetic acid. To 2.5 ml of distilled water, 1 ml of the above mixtures and 0.5 ml of Nessler's reagent were added. After keeping the mixture at 20 ^{0}C for 20 min, absorbance at 450 nm was measured with a UV-Vis spectrophotometer. The amount of released ammonia was determined. One IU of L-asparaginase is the amount of enzyme which liberates 1 µmol of ammonia per minute per ml [µmole/ml/min]. L-Glutaminase was assayed by using L-glutamine in place of L-asparagine.

3. RESULTS AND DISCUSSIONS

3.1. Plate screening

The organism had shown positive results for the plate screening. In the plate containing Asparagine as substrate, the color of the plate changed to pink upon incubation for 24hours. This is due to the release of ammonia by the L-asparaginase degradation of Asparagine into aspartic acid and ammonia. The same results were seen in plate with glutamine as substrate where the enzyme degraded Glutamine into glutamic acid and ammonia.

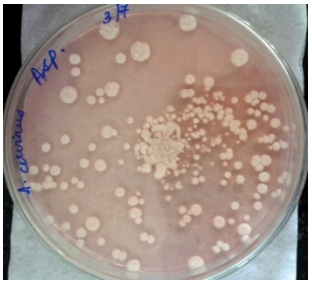


Fig. 1: *A. cervinus* showing positive result for Glutaminase.

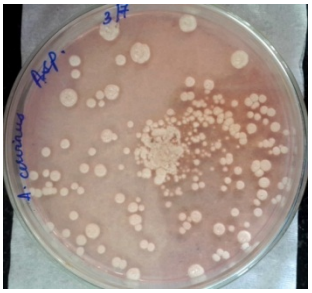
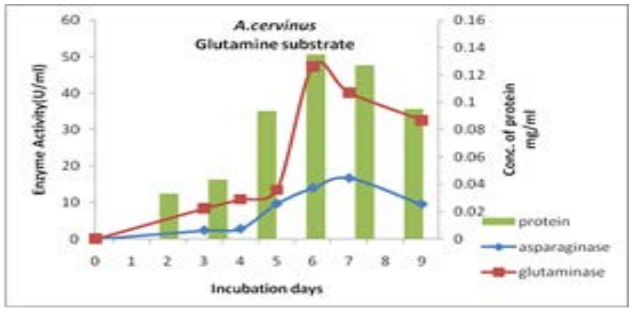


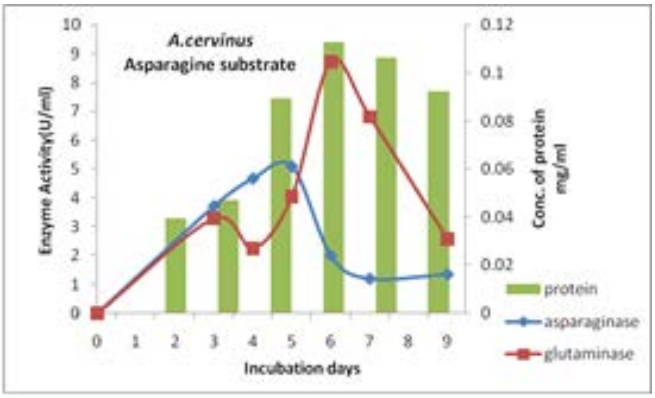
Fig. 2: *A. cervinus* showing positive result for Asparaginase.

3.2.Production in Submerged fermentation

Submerged fermentation was carried out for 9 days at 37^{0}C at 150 rpm. Every 24 hours 5ml of sample was collected. Submerged fermentation was carried out with two different substrates, Glutamine and Asparagine. Both the enzymes, L-Glutaminase and L-Asparaginase, were assayed in each flask. With Asparagine as substrate, activity of L-asparaginase was 5.08U/ml and L-glutaminase activity was 8.733U/ml. With Glutamine as substrate L-asparaginase activity was 16.65 U/ml and L-glutaminase activity was 47.38U/ml. In the presence of one substrate both the enzymes were produced because of the ability of the amino acids to convert from one amino acid to another[1]. The maximum activity was seen on the 6th day of incubation. Our results deviated from A.R. Soniyamby[10] who reported optimum incubation period as 4th day in liquid media. Protein was also determined by lowry's method. The results obtained are comparable with results obtained for *A. cervinus* [11].



Graph 1: Enzyme activity and protein content with Glutamine as substrate.



Graph 2: Enzyme activity and protein content with Asparagine as substrate.

4. CONCLUSION

From this study it was evident that *Aspergillus cervinus* is a potent source of both enzymes, L-Asparaginase and L-Glutaminase. The L-Asparaginase activity obtained was more than the activity reported by N. Sivagurunathan[11] in submerged media. Much work has not been done with *A.cervinus* as a producer of these enzymes. Hence, the organism can be explored and further optimization of media can be done to obtain higher enzyme activity.

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